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SHORT COMMUNICATION

Application of RAPD markers for characterization of γ-ray-induced rose mutants and assessment of genetic diversity

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Abstract Six parent and their 12 gamma ray-induced somatic flower colour mutants of garden rose were characterized to discriminate the mutants from their respective parents and understanding the genetic diversity using Random amplification of polymorphic DNA (RAPD) markers. Out of 20 primers screened, 14 primers yielded completely identical fragments patterns. The other 7 primers gave highly polymorphic banding patterns among the radiomutants. All the cultivars were identified by using only 7 primers. Moreover, individual mutants were also distinguished by unique RAPD marker bands. Based on the presence or absence of the 48 polymorphic bands, the genetic variations within and among the 18 cultivars were measured. Genetic distance between all 18 cultivars varied from 0.40 to 0.91, as revealed by Jaccard's coefficient matrix. A dendrogram was constructed based on the similarity matrix using the Neighbor Joining Tree method showed three main clusters. The present RAPD analysis can be used not only for estimating genetic diversity present in gamma ray-induced mutants but also for correct identification of mutant/new varieties for their legal protection under plant variety rights.

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Introduction

Mutation is a permanent genetic change which produces new forms. For a modern and industrialized floriculture, there is always demand and necessity for new varieties. Mutation breeding is now an established method for plant improvement. Mutation breeding has been most successful in roses in inducing novelties. The National Botanical Research Institute, Lucknow, India, undertook a project for the improvement of different vegetatively propagated ornamentals including rose, and has been very successful in producing quite a large number of new promising varieties by using gamma radiation (Datta 1989, 1992, 1995, 1997; Datta and Gupta 1982). The mutants are of direct use in the floriculture trade. However, most mutants deviate from the original variety only in minor characteristics and may thus be very difficult to distinguish genetically. Correct identification of new ornamental varieties is extremely important to protect plant breeders' rights for commercial exploitation. Accurate identification of plants is also desired for patent protection of propagated material. Chromosome numbers of horticultural varieties of rose have been detected by a number of workers (Meenakshi 1977; Lata 1977a, b). However, morphological observations and karyotype analysis of metaphase chromosomes have several limitations such as extensive evaluation time needed for assessment. Isozymes provide a limited number of informative markers and are affected by environmental variations. Use of present day molecular markers in addition to the classical methods provides more positive identification of new cultivars. Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), though

used for screening of genetic diversity, are laborious, usually involves radioactivity, and are not suited for routine application of cultivar identification. Random amplification of polymorphic DNA (RAPD) requires only small amounts of starting DNA, does not require prior DNA sequence information, and nor involves radioactivity (Williams et al. 1990), while data can be generated faster with less labor than other methods like RFLP. There are also some examples where an RAPD-based fingerprint technique has been used for mutant discrimination. Recently, 11 radiomutants from two chrysanthemum cultivars were characterized by RAPD (Kumar et al. 2006). This technique has also been used to study genetic variability in radiomutants from the lady group of chrysanthemum (Ruminska et al. 2004). Similarly, a mutated cherry was differentiated from its parental by 1 out of the 40 RAPD primers tested (Stockinger et al. 1996). But there is no information available on genetic diversity studies of mutant cultivars of rose evolved through induced mutagenesis. Therefore, the main objective of the present study was to discriminate the mutants from their respective parents and to understand the genetic diversity using RAPD.

Materials and methods

Plant material

Budwood of different rose cultivars were treated with 2, 3, and 4 krad of gamma rays, and eyes were budded on *Rosa indica* var. Odorata root stock. Mutation in flower color was detected in some treated plants in the form of chimera. The chimeric mutants have been established in pure form through vegetative propagation, and all the mutants are being maintained by vegetative propagation at the Floriculture Laboratory, National Botanical Research Institute, Lucknow, India (Datta 1997). For the present RAPD analysis, a number of somatic flower color mutants and their respective parents were selected. The details of the mutant and parent varieties are shown in Table 1 and Fig. 1.

DNA extraction

Total genomic DNA was extracted from young leaves of rose cultivars by the CTAB procedure Saghai-Maroof et al. (1984) with some modifications. Extraction in chloroform:isoamyl alcohol (24:1) followed by centrifugation twice at 14,000*g* helped to remove polysaccharides. RNA contaminants in all the samples were digested with 100 mg/ml RNase A for 30 min at 37°C, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1). After ethanol precipitation, DNA was resuspended in 100 ml of TE (10 mM Tris–HCl + 1 mM EDTA) buffer (pH 8.0). Average yield was calculated by a spectrophotometer (Ultraspec 2000; Pharmacia Biotech) and DNA samples were stored at −20°C.

PCR conditions

Twenty arbitrary decamer primers (Bangalore Genei, India) were used for polymerase chain reaction (PCR).

Table 1 Rose cultivars used in the study

Name of variety parent (P)/mutant (M)	Type	Color	Dose (gamma rays) (krad)	Year
'Contempo' (P)	Floribunda	Copper orange with yellow eye	–	–
'Contempo New' (M)	Floribunda	Pinkish red	3	–
'Contempo Pink' (M)	Floribunda	Pink	3	1986
'Contempo Stripe' (M)	Floribunda	Light yellow stripe on orange background	4	1983
'Contempo Tangerine' (M)	Floribunda	Tangerine orange	3	1984
'Contempo Yellow' (M)	Floribunda	Empire yellow	3	1984
'Imperator' (P)	Floribunda	Cherry red		
'Imperator Stripe' (M) (Twinkle)	Floribunda	Light pink stripe on cherry red background	3	1986
'Imperator Pink' (M)	Floribunda	Pink	3	1986
'First Prize' (P)	Hybrid tea	Blend of light red and deep pink		
'First Prize Lighter' (M)	Hybrid tea	Light pink	3	1989
'America's Junior Miss' (P)	Floribunda	Coral pink		
'Sukumari' (M)	Floribunda	Light pink	3	1984
'Sylvia' (P)	Hybrid tea	Pink		
'Sylvia White'	Hybrid tea	White	3	2002
'Mrinalini' (P)	Hybrid tea	Phlox pink		
'Mrinalini Lighter' (M)	Hybrid tea	Light pink	4	1989
'Mrinalini Stripe' (M)	Hybrid tea	White stripe on pink background	3	1991

Fig. 1 Gamma ray-induced mutants in rose. **a** 'Contempo' (P), **b** 'Contempo New', **c** 'Contempo Stripe', **d** 'Contempo Tangerine', **e** 'Contempo Yellow', **f** 'Imperator' (P), **g** 'Imperator Stripe', **h** 'Imperator Pink', **i** 'First Prize' (P), **j** 'First Prize Lighter', **k** 'American's Junior Miss', **l** 'Sukumari', **m** 'Mrinalini' (P), **n** 'Mrinalini Lighter', **o** 'Mrinalini Stripe'. *P* Parent

PCR reaction was performed in 20 ml reaction mixture containing 5 ng template DNA, 1 unit of Taq DNA polymerase, 100 μM dNTPs, 1.0 μM primer, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH-9.0), 50 mM KCl, 0.01% gelatin. PCR amplification was performed using a PTC-100 Peltier Thermal Cycler (MJ Research, USA) using the following conditions: preheating of 4 min at 94°C, 45 cycles of 15 s at 94°C, 45 s at 36°C and 1.5 min at 72°C and elongation was completed by a final extension of 4 min at 72°C. The final reaction mixture was cooled down to 4°C. After amplification, the PCR product was resolved by electrophoresis in 1% agarose gel with 1× TAE buffer. Bands were visualized by staining with ethidium bromide (0.5 μg/ml) under UV light and photographed. Only distinct bands were counted for data analysis, and faint bands were not considered. The size of the amplification products was estimated from a 100-bp DNA ladder (Sigma). All the reactions were repeated at least twice and only those bands reproducible on all runs were considered for analysis.

DATA analysis

DNA fragment profiles were scored in binary fashion with '0' indicating absence and '1' indicating presence of band. Genetic distance was calculated by Jaccard's coefficient (Jaccard 1908), which is as follows:

$$S_{ij} = N_{ij}/\left(N_{ii}+N_{ij}+N_{jj}\right)$$

where S_{ij} is the similarity index between the *i*th and *j*th genotype, N_{ij} is the number of bands present in both genotype, N_{ii} is the number of bands present in the *i*th genotype but absent in the *j*th genotype, and N_{jj} is the number of bands absent in the *i*th genotype and present in the *j*th genotype. The similarity matrix was converted to dissimilarity matrix ($1 - S_{ij}$), and a dendrogram was constructed using the Neighbor Joining Tree method using RAPDistance Package version 2.0 (Armstrong et al. 1994).

Results and discussion

Out of 20 primers screened, 14 primers yielded completely identical fragments patterns. The other 7 primers gave highly polymorphic banding patterns among the radiomutants (Table 2). The percentage of polymorphism varied from 50 (P31) to 100% (P4 and P34). PCR amplification with primer P4 clearly revealed that three bands (100, 200 and 400 bp) were absent in 'Contempo Stripe' as compared

Table 2 Sequences and codes of random decamers and the number of polymorphic fragments amplified

Primer	Sequence	Total number of bands	No. of polymorphic bands	% Polymorphism
P3	5′GACCGCTTGT3′	13	9	69.23
P4	5′GGGTAACGCC3′	12	12	100.00
P30	5′CTACGGAGGA3′	7	5	71.42
P31	5′CAGGCCCTTC3′	6	3	50.00
P33	5′GGCACTGAGG3′	5	3	60.00
P34	5′CAGCTCACGA3′	10	10	100.00
P40	5′AGCGTCCTCC3′	10	6	60.00
Total		63	48	76.19

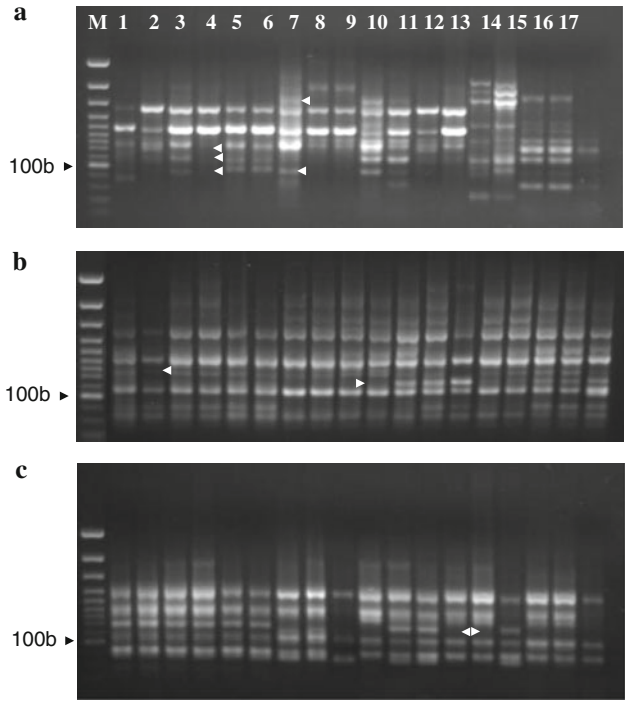

Fig. 2 Amplification of parent and mutant cultivars of rose using primer P4 (**a**), P40 (**b**), and P31 (**c**). *Lane 1* 'Contempo' (P), *2* 'Contempo New', *3* 'Contempo Pink', *4* 'Contempo Stripe', *5* 'Contempo Tangerine', *6* 'Contempo Yellow', *7* 'Imperator' (P), *8* 'Imperator Stripe', *9* 'Imperator Pink', *10* 'First Prize' (P), *11* 'First Prize Lighter', *12* 'American's Junior Miss', *13* 'Sukumari', *14* 'Sylvia' (P), *15* 'Sylvia White', *16* 'Mrinalini'(P), *17* 'Mrinalini Lighter', *18* 'Mrinalini Stripe'. *M* DNA molecular marker

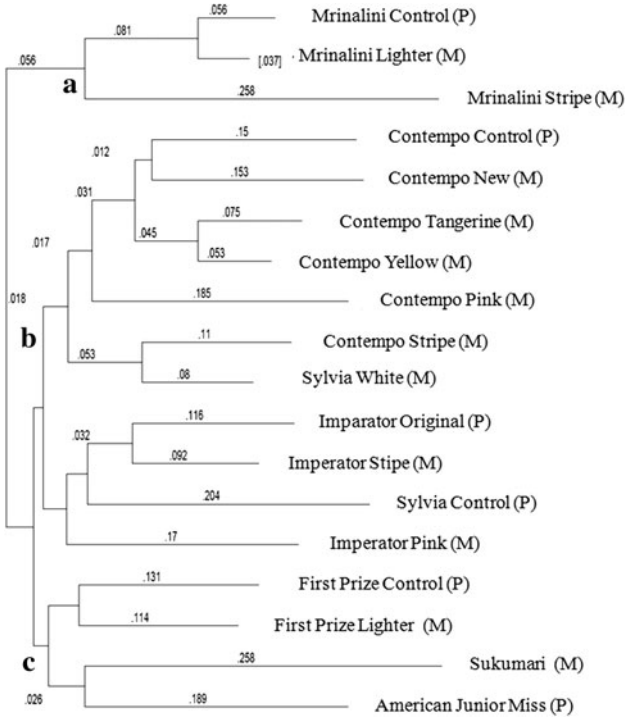

Fig. 3 Dendrogram of parent and mutant rose cultivars. 'Contempo' (P), 'Contempo New' (M), 'Contempo Pink' (M), 'Contempo Stripe' (M) 'Contempo Tangerine' (M), 'Contempo Yellow' (M), 'Imperator' (P), 'Imperator Stripe' (M), 'Imperator Pink' (M) 'First Prize' (P), 'First Prize Lighter' (M), 'American's Junior Miss' (P), 'Sukumari' (M), 'Sylvia' (P), 'Sylvia White' (M) 'Mrinalini' (P), 'Mrinalini Lighter' (M), 'Mrinalini Stripe' (M). *P* Parents *M* mutants

to the other 'Contempo' mutants (Fig. 2a, lane 4). Similarly, in 'Contempo New', a highly specific band of 350 bp was absent in comparison to other mutants when amplified with primer P40 (Fig. 2b, lane 2). In the case of 'Imperator' (parent), two highly specific bands (100 and 800 bp) were noticed when genomic DNA was amplified with P4 primer (Fig. 2a, lane 7). Similarly, a polymorphic band of 300 bp is absent in parent cultivar 'First Prize' (parent) but present in its mutant, when RAPD marker P40 was used (Fig. 2b, lane 10). Amplification with primer P31 indicated a highly distinct and polymorphic band (300 bp) present only in 'American's junior Miss', but absent in its mutant 'Sukumari', which can be used as a specific marker (Fig. 2c, lane 13–14). A highly polymorphic band (300 bp) is present in the mutant cultivar of 'Sylvia White' when the RAPD marker P31 was used (Fig. 2c, lane 15). Similarly, two highly distinct polymorphic bands (100 and 300 bp) are present in the parent cultivar 'Sylvia', which can be used to differentiate between the parent and its mutant when the RAPD marker P34 was used (data not shown). The parent cultivar 'Mrinalini' and its mutant 'Mrinalini Lighter' could not be distinguished by any primer tested. However,

Table 3 Similarity matrix of parent and mutant rose cultivars based on Jaccard's coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1.00																	
2	0.70	1.00																
3	0.59	0.58	1.00															
4	0.56	0.66	0.66	1.00														
5	0.72	0.75	0.71	0.67	1.00													
6	0.74	0.73	0.73	0.73	0.88	1.00												
7	0.66	0.66	0.58	0.62	0.64	0.69	1.00											
8	0.65	0.68	0.60	0.68	0.66	0.71	0.80	1.00										
9	0.61	0.60	0.57	0.64	0.58	0.60	0.60	0.77	1.00									
10	0.59	0.62	0.58	0.62	0.66	0.64	0.72	0.74	0.66	1.00								
11	0.60	0.60	0.66	0.66	0.71	0.73	0.66	0.68	0.71	0.66	1.00							
12	0.59	0.69	0.66	0.89	0.71	0.77	0.66	0.68	0.67	0.69	0.73	1.00						
13	0.50	0.54	0.44	0.54	0.55	0.60	0.60	0.56	0.44	0.60	0.55	0.54	1.00					
14	0.64	0.53	0.59	0.63	0.61	0.62	0.67	0.66	0.61	0.59	0.54	0.60	0.41	1.00				
15	0.55	0.46	0.57	0.51	0.56	0.57	0.58	0.56	0.62	0.67	0.62	0.54	0.60	0.62	1.00			
16	0.50	0.50	0.55	0.62	0.53	0.54	0.58	0.67	0.66	0.61	0.69	0.69	0.50	0.53	0.54	1.00		
17	0.52	0.52	0.58	0.62	0.56	0.54	0.62	0.67	0.66	0.64	0.69	0.65	0.50	0.59	0.60	0.91	1.00	
18	0.42	0.40	0.52	0.53	0.47	0.48	0.46	0.45	0.42	0.43	0.52	0.49	0.43	0.42	0.45	0.60	0.64	1.00

1 'Contempo' (P), *2* 'Contempo New', *3* 'Contempo Pink', *4* 'Contempo Stripe', *5* 'Contempo Tangerine', *6* 'Contempo Yellow', *7* 'Imperator' (P), *8* 'Imperator Stripe', *9* 'Imperator Pink', *10* 'First Prize' (P), *11* 'First Prize Lighter', *12* 'American's Junior Miss', *13* 'Sukumari', *14* 'Sylvia' (P), *15* 'Sylvia White', *16* 'Mrinalini'(P), *17* 'Mrinalini Lighter', *18* 'Mrinalini Stripe'

'Mrinalini Stripe' can be distinguished from its parent and 'Mrinalini Lighter' when RAPD marker P4 and P40 were used. Otherwise, some marginal variation for the fainter bands was noted for several primers studied. Earlier RAPD markers were tested for the identification of rose cultivars. All the cultivars were identified by using only three primers. Moreover, in accordance with our study, individuals were also distinguished by unique RAPD marker bands (Matsumoto and Fukui 1996).

Based on the presence or absence of the 48 polymorphic bands, the genetic variations within and among the 18 cultivars were measured (Fig. 3). The genetic distance between all 18 cultivars varied from 0.40 to 0.91, as revealed by Jaccard's coefficient matrix (Table 3). The dendrogram in Fig. 3 was constructed based on the similarity matrix using Neighbor Joining Tree method showed three main clusters. Cluster A consists of 'Mrinalini' and its mutants. Cluster B consists of two subclusters, one subcluster consisting of 'Contempo' and its mutants and the other containing 'Imperator' and its mutant. 'Sylvia' and its mutant have been placed in two different subclusters, indicating high genetic diversity from its parent which may be due to larger genomic rearrangements due to gamma irradiation. 'First Prize' and 'American's Junior Miss' and their mutants have been placed in cluster C. Cluster analysis separated rose mutants into different groups but the genetic distance observed between them was low. Similar observations have been reported earlier in chrysanthemum mutants (Kumar et al. 2006).

In the present study, most of the mutants appeared to be phenotypically the same as their parents, except for flower color. To clarify to what extent this phenotypical variation was related to the genetic level, the present RAPD analysis showed noticeable differences between parents and their mutants. This indicates that somatic flower color changes could have resulted due to some sort of genomic rearrangements rather than point mutations (Wolff et al. 1995) The present RAPD analysis can be used not only for estimating genetic diversity present in different floricultural crops but also for correct identification of mutant/new varieties for their legal protection under plant variety rights.

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